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(21) International Application Number: PCT/GB97/01065 (22) International Filing Date: 17 April 1997 (17.04.97) (30) Priority Data: 9607899.3 17 April 1996 (17.04.96) GB (71) Applicant (for all designated States except US): SCOTTISH CROP RESEARCH INSTITUTE [GB/GB]; Invergowrie, Dundee DD2 5DA (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): WILSON, Thomas, Michael, Aubrey [GB/GB]; The Coach House, 4 Balruddery Meadows, Invergowrie, Dundee DD2 5LJ (GB). CHAPMAN, Sean, Nicholas [GB/GB]; 47 Fort Street, Dundee DD5 2AB (GB). (74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: VIRUS-LIKE PARTICLE (57) Abstract Chimaeric pseudovirus particles and a method for producing a foreign protein using the same are disclosed. The pseudovirus particles comprise a protein (e.g. a coat protein) having a viral portion and a non-viral portion, and a nucleic acid (optionally chimaeric) to stabilize the aggregation of the protein, and create a helical ribonucleocapsid with the structure and symmetry approaching the native virus.			

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1 Virus-like particle.

2 This invention relates to a virus-like particle,
3 especially to a pseudovirus particle, and to a method
4 for the production of a chimaeric protein using such
5 virus-like particles. The protein can be a capsid
6 protein which can self assemble *in vivo* with the
7 nucleic acid (which may be chimaeric) to form the
8 virus-like particles.

9
10 Pseudovirus particles are virus-like particles
11 comprising viral coat protein subunits and a portion of
12 the wild-type viral nucleic acid. Pseudoviruses may
13 also include foreign nucleic acid. The coat protein
14 can be wild-type, modified or chimaeric. A pseudovirus
15 may lack at least a portion of the wild-type viral
16 nucleic acid (or may possess a non-functional analogue
17 of the wild-type nucleic acid) and this commonly
18 renders the pseudovirus incapable of some function
19 which is characteristic of the wild-type virus, such as
20 replication. Alternatively or additionally, other
21 genes may be missing or disabled, and the pseudovirus
22 may be, for example, replication competent but
23 incapable of cell-cell movement. The missing or
24 dysfunctional gene(s) can be provided on the genome of
25 a host cell or on a plasmid etc present in the host
26 cell, thereby restoring the function of the wild-type
27 virus to the pseudovirus when in the host cell.

28
29 The physical properties of the pseudovirus particle
30 such as shape, symmetry, nucleic acid:protein ratio are
31 usually similar to or identical with the wild-type

1 virus from which the pseudovirus is derived, although
2 particle length and width can be influenced by nucleic
3 acid length and coat protein composition respectively.
4

5 According to the present invention there is provided a
6 virus-like particle comprising nucleic acid and
7 protein, the protein having a first (viral) portion and
8 a second (non-viral) portion.
9

10 The term "virus-like particle" refers to self-
11 assembling particles which have a similar physical
12 appearance to virus particles and includes
13 pseudoviruses. Virus-like particles may lack or
14 possess dysfunctional copies of certain genes of the
15 wild-type virus, and this may result in the virus-like-
16 particle being incapable of some function which is
17 characteristic of the wild-type virus, such as
18 replication and/or cell-cell movement.
19

20 The nucleic acid can be DNA or RNA, according to the
21 genome of the virus from which the virus-like particle
22 is derived. The nucleic acid may comprise an origin-
23 of-assembly sequence (OAS) by which we mean a nucleic
24 acid sequence which permits initiation of assembly of
25 the protein and nucleic acid into virus-like particles.
26

27 Further according to the invention there is provided a
28 method of producing a protein having a first (viral)
29 portion and a second (non-viral) portion, the method
30 comprising expressing the protein in a cell, providing
31 a nucleic acid sequence capable of assembly with the
32 protein into a virus-like particle (VLP), and
33 permitting *in vivo* assembly of the protein and nucleic
34 acid into VLPs.
35

36 The virus-like particles can be purified from the cell

1 by standard techniques such as centrifugation etc, and
2 the chimaeric protein can optionally be cleaved to
3 release the second portion from the first portion, or
4 separated entirely from the nucleic acid. If the
5 chimaeric protein is left attached to the virus-like
6 particle, the whole virus-like particle can also be
7 used for presentation of peptide epitopes for
8 vaccination of animals, the production of therapeutic
9 or industrial proteins and polypeptides and/or the
10 delivery of therapeutic nucleic acid molecules
11 (optionally targeted delivery), such as ss or ds DNA or
12 RNA, including antisense molecules.

13
14 The nucleic acid can advantageously be provided from a
15 plasmid in the cell, possibly by transcription of such
16 a plasmid. The protein may be encoded by the same or
17 another plasmid in the cell. Alternatively, one or
18 both of the nucleic acid and protein can be coded from
19 the genome of the cell.

20
21 The cell is preferably a bacterium such as *E. coli*
22 although other forms of bacteria and other cells may be
23 useful, such as mammalian cells, plant cells, yeast
24 cells and insect cells. The cell may be a natural host
25 cell for the virus from which the virus-like particle
26 is derived, but this is not necessary.

27
28 The use of a cell for the assembly of the virus-like
29 particle *in vivo* enables facile cell handling
30 techniques to be employed to facilitate purification of
31 virus-like particles and purification of protein. In
32 addition, where it is desired to produce a second
33 portion protein which is toxic to some cells (eg plant
34 cells) a different (eg bacterial) cell may be employed.

35
36 The nucleic acid is preferably chosen in accordance

1 with its ability to assemble with the viral protein.
2 For example, the virus-like particle may be derived
3 from tobacco mosaic virus (TMV). In such a case, the
4 first portion of the protein is preferably derived from
5 TMV coat protein (CP), and the nucleic acid has at
6 least an OAS of eg 75 or more nucleotides derived from
7 TMV RNA. The sequence of the remainder of the nucleic
8 acid is not important, and it can be chosen to code for
9 the chimaeric protein or may be of some other eg
10 unrelated or therapeutic sequence. The inclusion of
11 nucleic acid in the virus-like particle means that the
12 particle is of helical symmetry and more stable than
13 simple aggregations of protein (eg planar, stacked or
14 helical arrays), which are normally created at low pH
15 *in vitro* from purified TMV coat protein, and can
16 dissociate outside a narrow pH range. Also, the length
17 of the particle can be selected by specifying a
18 particular length of nucleic acid. This results in a
19 more uniform range of particle sizes, which has
20 advantages in purification procedures such as
21 centrifugation, and in defining and regulating the
22 quality control for products for medical use.

23
24 A further advantage with the use of nucleic acid in the
25 assembly of virus-like particles is that the resultant
26 particle can have a regular multivalent and true
27 helical structure which can be more immunogenic than an
28 aggregation of protein or free subunits of protein.
29 The greater stability of the particle can also provide
30 longer access to the immune system in certain
31 embodiments.

32
33 The second portion of the chimaeric protein is
34 preferably disposed on the outer surface of the virus-
35 like particle. Thus where the particle is derived from
36 TMV, the second portion can be disposed on the amino or

1 carboxy terminus, or inserted in eg an internal loop
2 disposed on the outer surface of the CP. This can
3 result in improved assembly as compared with the
4 assembly of particles having a second portion on
5 another location of the CP, and can enhance immune
6 recognition of the second portion on the particle
7 surface, which is useful for embodiments where the CP
8 is an immunogen such as a vaccine. In certain cases it
9 may be possible to provide large second portion
10 proteins.

11
12 It is advantageous to use a virus which is flexuous (ie
13 which can bend easily) since chimaeric proteins with
14 large second portions may be able to assemble more
15 easily into virus particles which are flexuous than
16 those which are rigid. PVX is preferred since it forms
17 a flexuous particle.

18
19 A linker peptide can be incorporated between the first
20 and second portions and may have the function of
21 spacing the two portions from one another, reducing
22 steric restrictions. Optionally the linker peptide may
23 contain a cleavage site.

24
25 The term "cleavage site" refers to a short sequence of
26 amino acids which is recognisable and subsequently
27 cleavable by eg a proteolytic enzyme or by chemical
28 means. Suitable proteolytic enzymes include trypsin,
29 pepsin, elastase, factor Xa etc. Alternatively the
30 cleavage site may be vulnerable to cleavage by other
31 means, for example by addition of chemicals such as
32 cyanogen bromide (CNBr) or acids.

33
34 The term "cleavage site" may also include sequences
35 that self-leave such as the FMDV (Foot and Mouth
36 Disease Virus) 2A protease.

1 The cleavage site may be an integral part of either the
2 first or second portion. Hence either/or both of the
3 portions may include an integral cleavage site.

4
5 The second portion protein may be a short oligopeptide
6 (10-40 amino acids) or a relatively large polypeptide
7 eg over 10kDa. Proteins of 25-30 kDa may also be
8 suitable for production by the method of the invention.

9
10 The first (viral) portion of the chimaeric protein may
11 be any protein, polypeptide or parts thereof, derived
12 from a viral source including any genetically modified
13 versions thereof (such as deletions, insertions, amino
14 acid replacements and the like). In certain
15 embodiments the first portion will be derived from a
16 viral coat protein (or a genetically modified version
17 thereof). Mention may be made of the coat protein of
18 Potato Virus X as being suitable for this purpose.
19 Preferably the first portion has the ability to
20 assemble into virus-like particles by first-
21 portion/first portion association. Thus, a chimaeric
22 protein molecule can assemble with other chimaeric
23 protein molecules or with wild-type coat protein into a
24 chimaeric virion.

25
26 In a preferred embodiment of the invention the particle
27 is derived from a tobamovirus such as tobacco mild
28 green mosaic virus TMGMV), tobacco mosaic virus (TMV),
29 or from a potexvirus such as PVX, and in such an
30 embodiment, the second portion is preferably disposed
31 at or adjacent the N-terminus of the coat protein. In
32 PVX, the N-terminus of the coat protein is believed to
33 form a domain on the outside of the virion.

34
35 The second portion of the chimaeric protein may be any
36 protein, polypeptide or parts thereof, including any

1 genetically modified versions thereof (such as
2 deletions, insertions, amino acid replacements and the
3 like) derived from a source other than the virus from
4 which the first portion is derived. In certain
5 embodiments the second portion or the protein derived
6 therefrom is a biologically active or otherwise useful
7 molecule. The second portion or the protein derived
8 therefrom may also be a diagnostic reagent, an
9 antibiotic or a therapeutic or pharmaceutically active
10 agent. Alternatively the second portion protein may be
11 a food supplement.

12
13 It is not necessary for the first portion to comprise a
14 whole virus coat protein, but this remains an option.
15 Some no-essential amino acids could be removed during
16 construction of the CP gene.

17
18 The virus particle may be formed by the assembly of
19 chimaeric proteins only or by the mixed assembly of
20 chimaeric proteins together with some unmodified or
21 less modified forms of the naturally occurring wild-
22 type coat protein which forms the basis of the first
23 portion. For a mixed virus particle of the latter
24 type, there must be present polynucleotide(s) encoding
25 the chimaeric protein and the naturally occurring coat
26 protein. The appropriate protein-coding sequence(s)
27 may be arranged in tandem on the same molecule, or
28 could be generated by differential RNA splicing
29 Alternatively, the different proteins could be
30 translated from the same nucleotide sequence and
31 modified later, eg by *in vivo* processing such as self
32 cleavage. An example of this is the provision of a
33 chimeric CP gene encoding eg GFP-2A-CP fusion protein,
34 which is expressed from a single gene (eg on a plasmid,
35 from the genome of the cell, or from the RNA of the
36 VLP) and which self cleaves a variable number of the

1 translated proteins into separate GFP and CP, a
2 proportion of the translated proteins remaining
3 uncleaved as GFP-2A-CP. Thus a heterologous mixture of
4 CPs can be assembled into a VLP, with eg every 10th CP
5 bearing a second portion, and the remaining CPs being
6 cleaved, native (or substantially native) CPs. Thus
7 the potential problems with stearic hindrance which
8 might occur if all the CPs were chimaeric can be
9 overcome. Suitable co-translational cleavage sequences
10 can be chosen for particular cell types. The
11 efficiency of the co-translational cleavage can be
12 modified to produce the required proportion of
13 cleaved/whole CPs in the assembled VLP.

14
15 An advantage is gained by using a virus which forms a
16 helical particle with a relatively large pitch. PVX
17 has a pitch of 3.4nm and is to be preferred over
18 viruses with a lower pitch. Virus particles with
19 higher pitches may be able to accommodate larger
20 protein insertions on their surfaces since their coat
21 proteins assemble with more space between them than
22 coat proteins of viruses with lower pitches.

23
24 The method can be used for expression of metabolic
25 enzymes for pathway engineering, nutritional
26 supplements (eg hi-met proteins), anti-potato cyst
27 nematode lectins, gut protease inhibitors, anti-
28 botrytis agents, PGIPs, anti-insect *Bacillus*
29 *thuringiensis* toxin and herbicide resistance agents,
30 industrial enzymes, pharmaceuticals, therapeutic
31 proteins and nucleic acids, and as bioreactors.

32
33 While modifications and improvements may be
34 incorporated without departing from the scope of the
35 invention, embodiments will now be described by way of
36 the following examples and with reference to the

1 accompanying drawings in which:

2

3 Fig 1 is a schematic representation of the plasmid
4 pA27;

5 Fig 2 is an SDS PAGE analysis of proteins from purified
6 TMV and pseudovirions. Samples were electrophoresed on
7 an SDS/PAGE gel and silver stained. Lane 1, purified
8 TMV. Lane 2, VLPs purified from E. coli BL21(DE3)
9 cells transformed with plasmids pA27 and pLys102. The
10 positions of coelectrophoresed marker proteins and
11 their molecular weights in kDa are shown to the left;
12 Fig 3 is an electron microscope image of VLPs. VLPs
13 purified from E. coli BL21(DE3) cells transformed with
14 plasmids pA27 and pLys102 were negatively stained with
15 2% sodium phosphotungstate pH 5.0 and viewed in the
16 electron microscope. Magnification x 20,000;
17 Fig 4 shows sequence information for LITMUS 39 plasmids
18 used in Example 2;

19 Fig 5 shows a schematic representation of cDNA
20 constructs used in Example 2;

21 Fig 6 shows immunoblot analysis of extracts of leaves
22 probed with anti-CP antiserum; and

23 Fig 7 shows immunoblot analysis of virus prepared from
24 plants infected with a VLP.

25

26 Example 1:

27 A sequence encoding two glycine residues and an eight
28 amino acid antigenic epitope (EQPTTRAQ) from VP1 of
29 poliovirus type 3 [1] was fused to the 3' end of a
30 synthetic gene coding for the tobacco mosaic virus
31 (TMV) coat protein by PCR amplification with mutagenic
32 primers. The plasmid pTMVCP [1] was used as a template
33 for amplification with primers P1311 (5' AAG-AAT-TCA-
34 TAT-GTC-TTA-TTC-GAT-TAC-C 3') and P1312 (5' AAG-GAT-
35 CCT-CAC-TGA-GCA-CGA-GTA-GTC-GGC-TGT-TCA-CCA-CCA-GTT-
36 GCC-GGG-CCC-GAG 3'). The amplification product was

1 treated with T4 DNA polymerase to make it blunt-ended
2 and ligated into *EcoRV* digested pKR [2]. The ligation
3 products were transformed into *E. coli* strain JM101.
4 Transformants were screened for the desired plasmid,
5 pA11, containing the gene encoding the modified TMV
6 coat protein.

7
8 To enable expression of the modified TMV coat protein
9 in *E. coli* a fragment encompassing the modified gene
10 was cloned into an expression vector, under the
11 transcriptional control of T7 promoter and T₀
12 terminator sequences. The plasmid pA11 was digested
13 with *NdeI* and *BamHI* and the 510 base pair fragment
14 released was cloned between the same sites of pET3a
15 [3]. The nucleotide sequence of the resulting plasmid,
16 pA27 (Figure 1), in the region encoding the eight amino
17 acid epitope and the linker of two glycine residues,
18 was confirmed by nucleotide sequence determination.

19
20 In Figure 1, sequence encoding TMV coat protein and ten
21 amino acid peptide fused to the carboxy-terminus are
22 indicated by boxes marked TMV CP and PEP respectively.
23 Restriction endonuclease sites used for the
24 introduction of the modified TMV coat protein gene into
25 the plasmid pET3a are indicated above. The T7 promoter
26 and T₀ terminator sequences from the plasmid pET3a are
27 indicated by a double thickness arrow and line
28 respectively. The nucleotide sequence of the 3' end of
29 the modified TMV coat protein gene and the amino acids
30 encoded by this sequence are shown below. The
31 nucleotide sequence encoding the additional ten amino
32 acids and the amino acids themselves are shown in bold.

33
34 To obtain expression of the modified TMV coat protein
35 and production of pseudovirions the plasmid pA27 was
36 transformed into *E. coli* BL21(DE3) cells that had

1 previously been transformed with the plasmid pLys102
2 [4]. The plasmid pLys102 produces a chimaeric RNA
3 transcript encoding chloramphenicol acetyl transferase
4 and containing the TMV origin-of-assembly sequence,
5 which when co-synthesized with TMV coat protein in *E.*
6 *coli* directs the assembly of pseudovirus particles of
7 70nm length (modal) and 18nm diameter. That plasmid
8 pA27 directed the synthesis of modified TMV coat
9 protein was confirmed by SDS/PAGE analysis of IPTG
10 induced bacterial lysates [4]. Production of a TMV coat
11 protein-related protein with a slightly lower mobility
12 than unmodified TMV coat protein was detected by
13 Coomassie blue staining and immunoblotting of SDS/PAGE
14 gels as described by Hwang et al. [4].

15
16 VLPs containing the modified TMV coat protein were
17 purified using a protocol based on that described by
18 Hwang et al. [4]. Colonies of BL21(DE3) co-transformed
19 with pA27 and pLys102 were used to inoculate 5 ml of
20 M9ZB medium supplemented with 100 µg/ml ampicillin and
21 35 µg/ml chloramphenicol. Cultures were grown overnight
22 at 37°C. The bacteria were pelleted from the overnight
23 cultures and used to inoculate 500 ml of M9ZB medium
24 supplemented with ampicillin and chloramphenicol. The
25 large-scale cultures were grown at 37°C until mid-log
26 phase ($A_{600} = 0.7$). Cultures were induced with 0.4 mM
27 IPTG and incubated at 30°C for eighteen hours. Cells
28 were harvested by centrifugation (4800 x *g*, 4°C, 6
29 min). Bacterial pellets were resuspended in 3ml of TE
30 (10 mM Tris-HCl pH 7.5 / 1 mM EDTA) and incubated with
31 lysozyme (0.4 mg/ml) at 20°C for 60 min. Bacteria were
32 lysed by addition of 4 ml 40% w/v sucrose in TE and
33 then 16 ml of TE. DNase I was added to 6.5 µg/ml and
34 the lysates incubated at 37°C for 90 min. Bacterial
35 debris was removed by centrifugation (20800 x *g*, 4°C,
36 30 min). The resulting supernatants were extracted with

1 10 ml of chloroform and the two phases separated by
2 centrifugation (9200 x g, 4°C, 10 min). 3.7 ml of 5M
3 NaCl and 2.63 ml of 40% polyethylene glycol (average
4 molecular weight 6000) were added to 20 ml of the
5 aqueous phase. The solutions were mixed and incubated
6 on ice for 60 min. Precipitated material was collected
7 by centrifugation (20800 x g, 4°C, 15 min). The
8 pelleted material was resuspended in 1 ml of TE.
9 Insoluble material was removed by centrifugation (16000
10 x g, 4°C, 5 min). The supernatant was centrifuged
11 (160000 x g, 4°C, 120 min) on a sucrose gradient (10-
12 40% w/v in TE). Fractions were collected from the
13 gradients and those containing helical TMV-like
14 particles, assessed by double-antibody sandwich ELISA
15 with a mouse monoclonal antibody specific for an
16 epitope in the TMV coat protein helix as described by
17 Hwang et al. [4], were pooled for further purification.
18
19 VLPs were collected by centrifugation (235,000 x g,
20 15°C, 150 min). Pelleted pseudovirions were resuspended
21 in 0.5 ml of TE. Insoluble material was removed by
22 centrifugation (840 x g, 4°C, 5 min). The supernatant
23 was centrifuged (189,000 x g, 15°C, 120 min) on a CsCl
24 gradient (10-40% (wt/wt) in TE). Bands containing
25 pseudovirus were collected from the gradients and
26 dialyzed against 50 mM sodium phosphate pH 7.0.
27
28 The yield of VLPs was estimated by measuring the
29 absorption at 260 nm. The final yield of pseudovirus
30 was 5.8 mg from 500 ml of culture. The purity of the
31 pseudovirus preps was assessed by silver staining of
32 samples electrophoresed on SDS/PAGE gels (Figure 2). On
33 SDS/PAGE gels the unmodified TMV coat protein produced
34 by pET302 and the modified coat protein produced by
35 pA27 migrate relative to protein standards (Bio-Rad)
36 with apparent molecular weights of 20.9 kDa and 22.6

1 kDa respectively. The predicted molecular weights for
2 these two proteins are 17.7 kDa and 18.6 kDa
3 respectively.

4
5 The integrity of the pseudovirus preparations was
6 assessed by negative staining of pseudovirus samples
7 with 2% sodium phosphotungstate and observation of the
8 stained samples in the electron microscope (Figure 3).
9 Pseudovirus preparations were diluted to 1 mg / ml in
10 50 mM sodium phosphate pH 7.0 for immunization of mice.

11
12 Example 2:

13 A plasmid containing the tobacco mild green mosaic
14 virus (TMGMV) coat protein (CP) gene and 3'
15 untranslated region (UTR) was produced to facilitate
16 the production of green fluorescent protein (GFP),
17 foot-and-mouth disease virus 2A, TMGMV CP gene fusions.
18 A 955 base pair (bp) fragment containing the TMGMV CP
19 and 3' UTR was PCR amplified from the plasmid 30B (W.O.
20 Dawson, Citrus Research and Education Center) using the
21 primers TMGMV-CP-Apa (5' CAA-TGG-GCC-CTA-TAC-AAT-CAA-
22 CTC-T 3') and M13-Reverse (5' AGC-GGA-TAA-CAA-TTT-CAC-
23 ACA-GGA 3'). The primer TMGMV-CP-Apa was designed to
24 mutagenize the sequence coding for the initiating
25 methionine and first proline codon of the TMGMV CP to
26 an ApaI restriction enzyme site. This results in the
27 conversion of the methionine codon to a glycine codon,
28 but maintains the proline codon. The 837bp fragment
29 released by digestion of the PCR amplification product
30 with the restriction endonucleases ApaI and KpnI was
31 cloned into the 3322bp fragment released by digestion
32 of pSL1180 (Pharmacia) digested with the same
33 restriction endonucleases and treated with calf
34 intestinal alkaline phosphatase. The resulting plasmid
35 was named pSL.TMGMV-CP-UTR.

36

1 CFP-2A-TMG MV CP gene fusions were produced by cloning
2 DNA fragments containing GFP-2A fusions into pSL.TMG MV-
3 CP-UTR adjacent to the codon for the first proline in
4 the TMG MV CP gene. A selection of LITMUS 39 (New
5 England Biolabs) based plasmids containing GFP-2A-
6 potato virus X CP gene fusions were used as sources for
7 the GFP-2A gene fusion.

8
9 The nucleotide sequence and amino acids encoded by the
10 different LITMUS 39 based plasmids between the carboxy-
11 terminal lysine codon of the GFP gene and the amino-
12 terminal proline codon of the PVX CP gene are shown in
13 Figure 4.

14
15 These plasmids contain a variety of sequences coding
16 for different 2A amino acid sequences between the
17 carboxy-terminal lysine codon of GFP and the first
18 proline codon of the PVX CP. Fragments of between 900
19 and 1050bp were PCR amplified from the plasmids pLit,
20 GFP-2A₁₆₈-CP, pLit.GFP-2A₁₆₈-CP, pLit.GFP-2A₂₃₈-CP and
21 pLit.GFP-2A₃₈₈-CP using the primers GFP-5'-Sal (5' TCA-
22 ATC-GTC-GAC-ATG-AGT-AAA-GGA-GAA-GAA 3') and N3#4 (5'
23 TGT-ACT-AAA-GAA-ATC-CCC-ATC-C 3'). The primer GFP-5'-
24 Sal introduces a SalI restriction enzyme site upstream
25 of the initiating methionine codon of the GFP gene.
26 Fragments containing the GFP gene fused to the
27 different 2A sequences were released by digestion of
28 the PCR amplification products with SalI and ApaI and
29 ligated into the large fragment released by digestion
30 of pSL.TMG MV-CP-UTR with the same restriction enzymes
31 and treated with calf intestinal phosphatase. The
32 resulting plasmids were digested with SalI and BstEII
33 and the released fragments containing the GFP-2A-TMG MV
34 CP gene fusion and TMG MV UTR were introduced into the
35 plasmid 30B digested with XhoI and BstEII to regenerate
36 full-length TMV based clones. Thus the final clones

1 comprise wild-type TMV strain U1 sequence up to
2 position 5757 in the CP gene, with the exception of a
3 mutagenized CP initiating methionine codon, followed by
4 a short polylinker sequence, the GFP-2A-TMG MV CP gene
5 fusions and the TMGMV 3' UTR.

6
7 Figure 5 shows a schematic representation of viral cDNA
8 constructs used in this example. Boxes represent
9 coding sequences. The genes for the three viral
10 proteins common to all constructs are indicated by
11 their predicted Mr values (K=kDa). The genes for the
12 green fluorescent protein, 2A oligopeptide and TMGMV CP
13 are indicated by GFP, 2A and CP respectively.
14 Restriction enzyme sites used in the cloning procedures
15 are indicated above.

16
17 *In vitro* run-off transcripts were synthesized from KpnI
18 linearized plasmids p30B.GFP-2A₁₆₈-CP, p30B.GFP-2A_{16K}-CP,
19 p30B.GFP-2A₂₃₈-CP, p30B.GFP-2A_{38K}-CP and p30B.GFP, a
20 derivative of p30B that has had the GFP gene introduced
21 into the unique XhoI site of P30B, which expresses free
22 GFP. The transcripts derived from all the plasmids
23 were infectious when inoculated onto *Nicotiana*
24 *benthamiana* plants; virus derived from transcript-
25 infected plants is referred to subsequently by the name
26 of the progenitor plasmid without the "p" prefix.
27 Following inoculation, all the viruses caused the
28 development of fluorescent regions which were first
29 detectable by eye under UV illumination between three
30 and four days post inoculation. Subsequent long
31 distance movement of the virus led to the appearance of
32 green fluorescence in systemically infected leaves.
33 The appearance of fluorescence in systemically infected
34 leaves occurred at a similar time, nine days post
35 inoculation, for plants infected with 30B.GFP, 30B.GFP-
36 2A₁₆₈-CP and 30B.GFP-2A_{16K}-CP, but was delayed for

1 30B.GFP-2A₂₃₈-CP and 30B.GFP-2A_{38K}-CP.

2

3 Western blotting of protein extracts from systemically
4 infected *N. benthamiana* leaves, probed with rabbit
5 polyclonal antisera raised against TMV CP (Figure 6),
6 detected two protein species in each of the 30B.GFP-2A-
7 CP infected samples. This result indicated that the
8 modified viruses were producing a GFP-2A-CP fusion
9 protein, the *in vivo* processing of which resulted in
10 the production of a GFP-2A fusion protein and free
11 TMGMV CP. For 30B.GFP-2A₁₆₈-CP, 30B.GFP-2A_{16K}-CP and
12 30B.GFP-2A_{38K}-CP the majority of CP related protein
13 produced was in the unfused form. Protein was prepared
14 from mock-inoculated control plants (lane 1) or from
15 plants inoculated with *in vitro* transcripts synthesized
16 from plasmid DNAs (p30B.GFP, lane 2; p30B.GFP-2A₂₃₈-CP,
17 lane 3; p30B.GFP-2A₁₆₈-CP, lane 4; p30B.GFP-2A_{16K}-CP, lane
18 5; p30B.GFP-2A_{38K}-CP, Lane 6). Lane 7 contains 125ng of
19 TMGMV CP. The predicted Mr values of TMGMV CP, GFP and
20 GFP-2A-CPs are 17.5 kDa, 26.9 kDa and between 46 and 52
21 kDa, respectively. The Mr values of standards (X10₃)
22 are shown on the left.

23

24 The observation that the modified viral constructs were
25 capable of rapid systemic movement like 30B.GFP
26 suggested that they were also capable of virus particle
27 formation. To confirm that this was the case
28 homogenates were prepared by grinding fluorescent
29 inoculated leaf tissue from plants infected with
30 30B.GFP and 30B.GFP-2A₂₃₈-CP in a "mini-mortar" with
31 50mM phosphate buffer pH 6.5. The homogenates were
32 applied to a carbon coated grid and stained with 2%
33 sodium phosphotungstate pH 6.5 prior to observation in
34 the electron microscope. 30B.GFP-2A₂₃₈-CP was found to
35 produce rod-shaped particles like those produced by
36 30B.GFP. To test whether the particles produced by

1 30B.GFP-2A₂₃₈-CP had incorporated GFP-2A-CP fusion
 2 protein as well as free TMGMV CP immunotrapping
 3 (Roberts 1986, in Electron microscopy of proteins,
 4 Academic Press) was performed with rabbit polyclonal
 5 antisera raised against GFP and TMV.CP. While 30B.GFP
 6 infected tissue showed enhanced trapping with the TMV-
 7 CP antisera, but not with the GFP antisera, 30B.GFP-
 8 2A₂₃₈-CP infected tissue showed enhanced trapping with
 9 both antisera (Table 1). This result suggested that
 10 the modified virus was capable of incorporating GFP-2A-
 11 CP fusion protein into particles.

12
 13 Table 1
 14 Number of particles/1000 μ m²

15 16 Coating 17 antiserum	15	
	30B.GFP	30B.GFP-2A ₂₃₈ -CP
18 None	223 +/- 57.0	3.5 +/- 1.33
19 TMV CP	4690 +/- 1200	58.0 +/- 3.16
20 GFP	112 +/- 9.45	67.5 +/- 15.2

21 To confirm this a virion extraction (Kearney et al, in
 22 Plant Molecular Biology Manual L1:1-16, Kluwer Academic
 23 Publishers) was performed on fluorescent, systemically
 24 infected tissue of plants infected with 30B.GFP-2A₁₆₈-
 25 CP. Western blot analysis (Fig 7) of the virus
 26 preparation with GFP (B) and TMV CP (A) antisera
 27 demonstrated that the virus contained TMGMV CP and CGP-
 28 2A-CP fusion protein but no GFP-2A fusion protein. Mr
 29 values shown on left of Fig 7 ($\times 10^{-3}$) Thus the GFP-2A-
 30 CP fusion protein was assembled with free TMGMV CP into
 31 virus particles.

32
 33
 34
 35
 36

- 1 Modifications and improvements can be incorporated
- 2 without departing from the scope of the invention.
- 3

1 Documents incorporated herein by reference:

2

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14 [4] Hwang, D.-J., Roberts, I.M. and Wilson, T.M.A.
15 (1994). Proceedings National Academy of Sciences
16 U.S.A., 91, 9067-9071. WO 94/10329 (Rutgers
17 University), see particularly deposit information
18 therein.

1 Claims:

2

3 1 A method of producing a protein having a first
4 (viral) portion and a second (non-viral) portion, the
5 method comprising expressing the protein in a cell,
6 providing a nucleic acid sequence capable of assembly
7 with the protein into a virus-like particle (VLP), and
8 permitting *in vivo* assembly of the protein and nucleic
9 acid into VLPs.

10

11 2 A method as claimed in claim 1, wherein the VLPs
12 are subsequently purified from the cell.

13

14 3 A method as claimed in claim 1 or claim 2, wherein
15 after assembly the protein is cleaved to release the
16 second portion from the first portion, or is separated
17 entirely from the nucleic acid.

18

19 4 A method as claimed in any preceding claim,
20 wherein the nucleic acid is provided from a plasmid.

21

22 5 A method as claimed in claim 4, wherein the
23 protein is encoded by the same or another plasmid in
24 the cell, or from the genome of the cell.

25

26 6 A method as claimed in any preceding claim,
27 wherein the cell is selected from bacterial cells,
28 mammalian cells, plant cells, yeast cells and insect
29 cells.

30

31 7 A method as claimed in claim 6, wherein the cell
32 is a natural host cell for the virus from which the
33 virus-like particle is derived.

34

35 8 A method as claimed in any preceding claim,
36 wherein the second portion of the protein is disposed

1 on the outer surface of the VLP.

2

3 9 A method as claimed in any preceding claim,
4 wherein the VLP is flexuous.

5

6 10 A method as claimed in any preceding Claim
7 wherein a cleavage site is incorporated on one of, or
8 between, said first and second portions.

9

10 11 A method as claimed in any preceding claim,
11 wherein a linker peptide is incorporated between the
12 first and second portions.

13

14 12 A method as claimed in any preceding claim,
15 wherein the second portion has a molecular weight of up
16 to 10 kDa.

17

18 13 A method as claimed in any one of claims 1 to 11,
19 wherein the second portion has a molecular weight of
20 between 10 kDa and 30 kDa.

21

22 14 A method as claimed in any one of claims 1 to 11,
23 wherein the second portion has a molecular weight over
24 30kDa.

25

26 15 A method as claimed in any preceding claim,
27 wherein the first portion is derived from a viral coat
28 protein or a modified version thereof.

29

30 16 A method as claimed in any preceding claim,
31 wherein the first portion is derived from a tobamovirus
32 or a potexvirus.

33

34 17 A method as claimed in any preceding claim,
35 wherein the second portion or the protein derived
36 therefrom is a biologically or pharmaceutically active

1 or useful molecule.

2

3 18 A method as claimed in any one of claims 1 to 16,
4 wherein the second portion or the protein derived
5 therefrom is a diagnostic reagent.

6

7 19 A method as claimed in any one of claims 1 to 16,
8 wherein the second portion or the protein derived
9 therefrom is a food supplement.

10

11 20 A method as claimed in any preceding claim,
12 wherein the virus particle is formed by a mixed
13 assembly of chimaeric proteins together with some
14 unmodified or less modified forms of the naturally
15 occurring wild-type coat protein which forms the basis
16 of the first portion.

17

18 21 A method as claimed in claim 20, wherein the
19 chimaeric proteins and the unmodified or less modified
20 forms of the naturally occurring wild-type protein are
21 expressed from different sequences of nucleic acid.

22

23 22 A method as claimed in claim 21, wherein the
24 different sequences are on the same piece of nucleic
25 acid in the cell.

26

27 23 A method as claimed in claim 21, wherein the
28 different sequences are on different pieces of nucleic
29 acid in the cell.

30

31 24 A method as claimed in claim 20, wherein the
32 chimaeric proteins and the unmodified or less modified
33 forms of the naturally occurring wild-type protein are
34 expressed from the same sequence of nucleic acid.

35

36 25 A method as claimed in claim 24, wherein the

1 chimaeric proteins and the unmodified or less modified
2 forms of the naturally occurring wild-type protein are
3 generated by co-translational modification, or are
4 modified after translation.

5

6 26 A method as claimed in any preceding claim,
7 wherein the virus from which the first portion is
8 derived forms a particle with a relatively high pitch
9 of helix.

10

11 27 A virus-like particle (VLP) comprising nucleic
12 acid and protein, the protein having a first (viral)
13 portion and a second (non-viral) portion.

14

15 28 A VLP as claimed in claim 27 wherein the nucleic
16 acid comprises an origin of assembly sequence which
17 permits initiation of assembly of the protein and
18 nucleic acid into VLPs.

19

20 29 A VLP as claimed in either of claims 27 or 28,
21 wherein the second portion (non-viral) of the protein
22 is disposed on the outer surface of the VLP.

23

FIG. 1

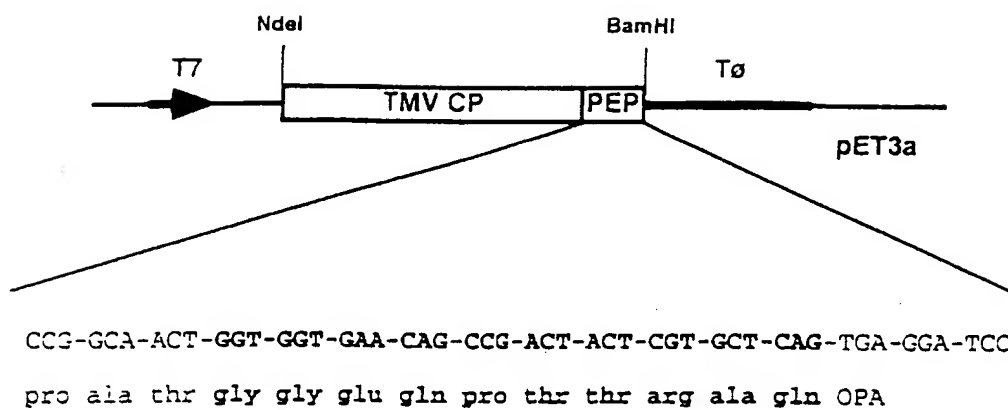


FIG. 2

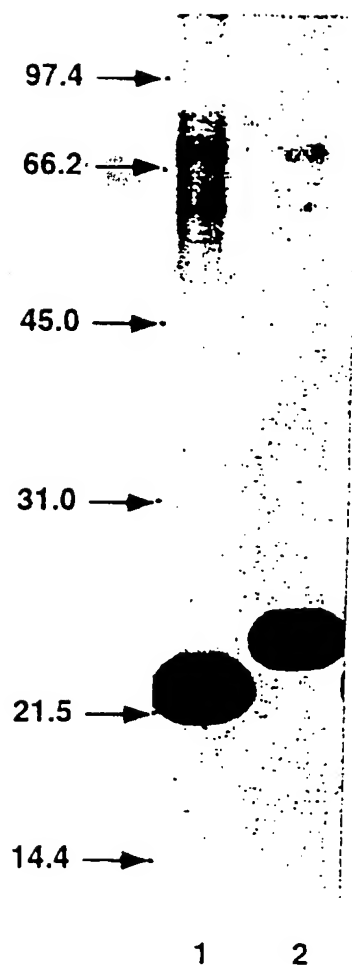


FIG. 3

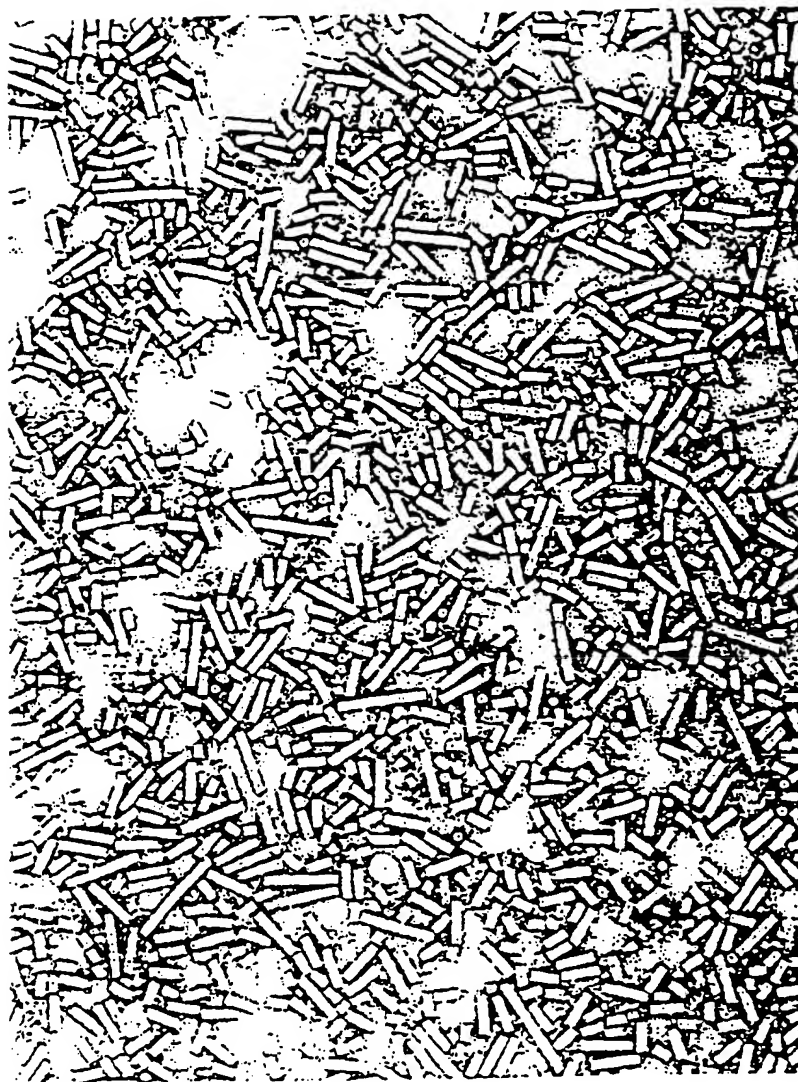


Figure 4.pLit.GFP-2A_{23H}-CP

TCC GGA TCT AGA GCA CCT GTG AAA CAG CTG TTG AAT TTT GAC CTT CTT AAG CTT GCG GGA GAC GTC GAG TCC AAC CCT GGG
 S G S R A P V K Q L L H F D L L L K L A G D V E S H P G.

pLit.GFP-2A_{16H}-CP

TCC GGA TCT AGA AAT TTT GAC CTT CTT AAG CTT GCG GGA GAC GTC GAG TCC AAC CCT GGG
 S G S R H F D L L L K L A G D V E S H P G.

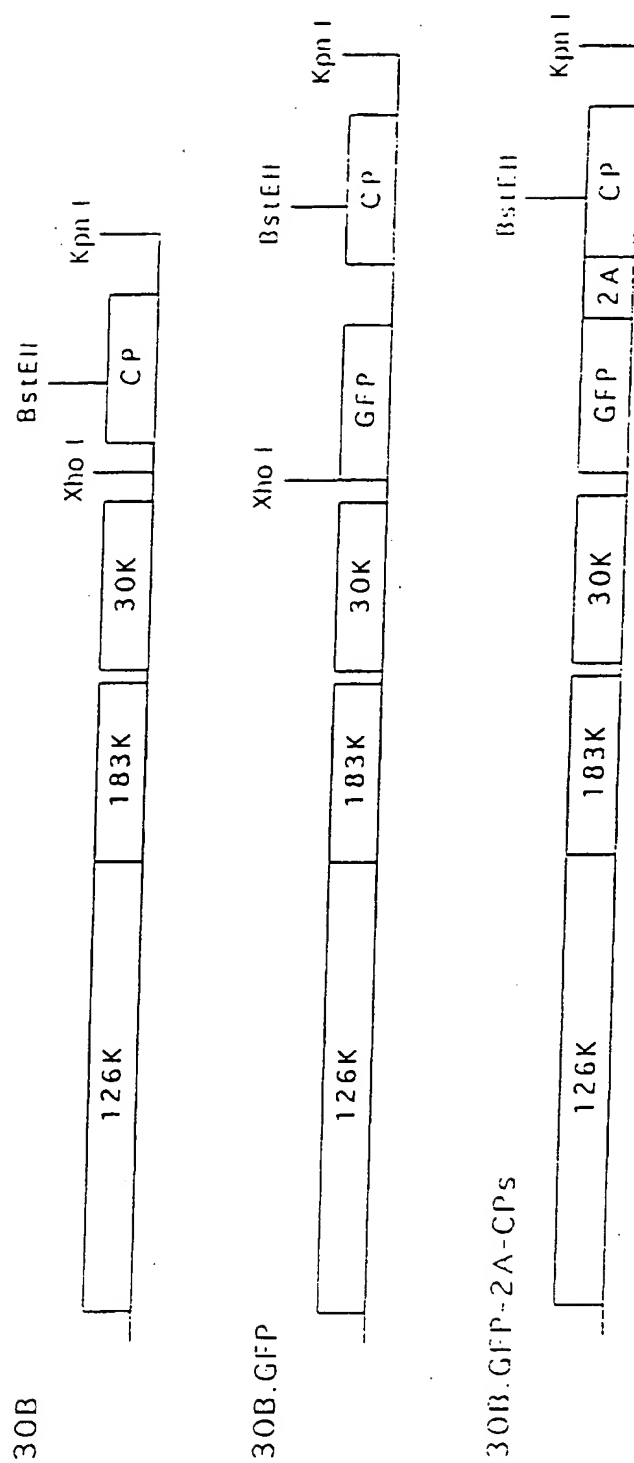
pLit.GFP-2A_{14H}-CP

TCC GGA TCT AGA AAT TTT GAC CTT CTT AAG CTT GCG GGA GAC GTC GAG TCC AAC CCT GGG
 S G S R H F D L L L K L A G D V E S H P G.

pLit.GFP-2A_{50H}-CP

TCC GGA TCT AGA GTC ACC GAG TTG CTT TAC CCG ATG AAG AGG GCC GAA ACA TAC TGT CCA AGC CCC TTG CTG GCA ATC CAC GCA ACT GAA GCC
 S G S R V T E L L Y R H K R A E T Y C P R P L L A I H P T E A.
 ACA CAG AAA CAG AAA AAT GTG CCA CCG GTG AAA CAG ACT TTG AAT TTT GAC CTT CTC AAG TTG GCG GGA GAC GTC GAG TCC AAC CCT GGG
 R H K Q K I V A P V K Q T L H F D L L L K L A G D V E S H P G.

Figure 5.



5/7

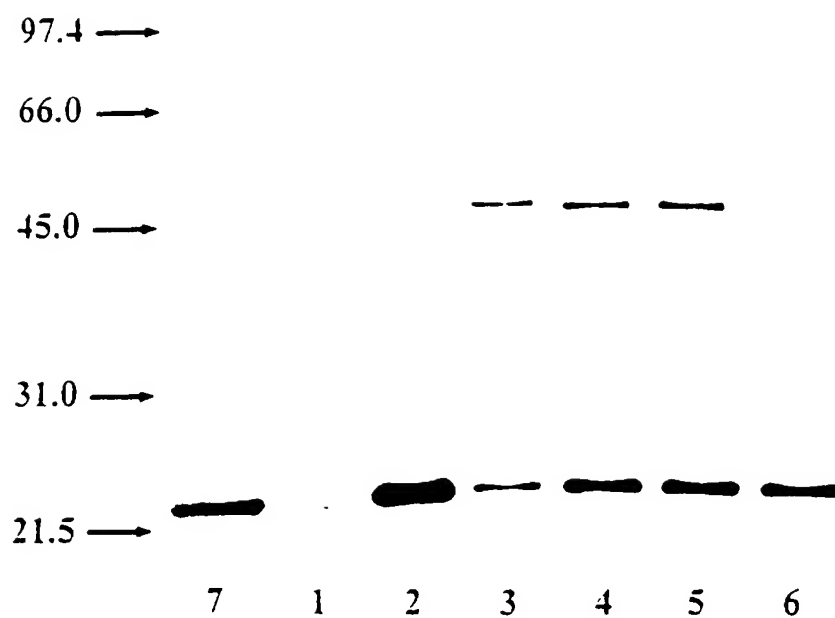
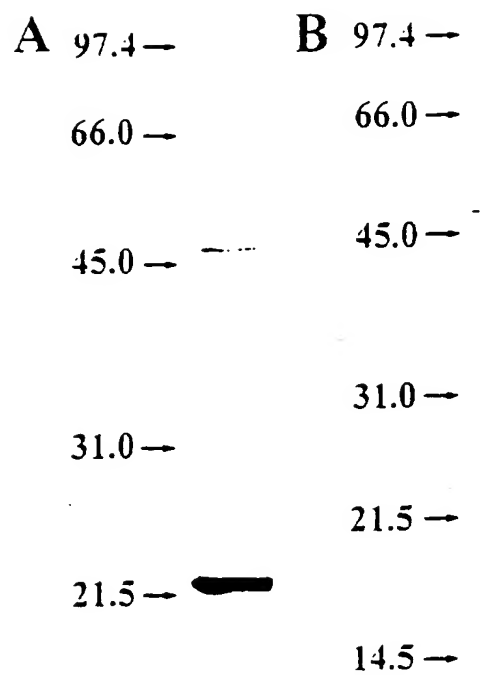
Figure 6.

Figure 7.

INTERNATIONAL SEARCH REPORT

Internat. Application No
PCT/GB 97/01065

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C12N15/82 C12P21/02 C12N7/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M.N. JAGADISH ET AL.: "High level production of hybrid Potyvirus-like particles carrying repetitive copies of foreign antigens in Escherichia coli" BIO/TECHNOLOGY., vol. 11, no. 10, October 1993, NEW YORK US, pages 1166-1170, XP002040652 see the whole document ---	1-6, 8-12, 15-18, 20-29
X	WO 96 05292 A (CONNAUGHT LABORATORIES LIMITED) 22 February 1996 see page 7, line 25 - page 12, line 27; figures 1-16 ---	1-12, 15, 17, 20-29
X	WO 95 10624 A (BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 20 April 1995 see the whole document -----	1-12, 15, 17, 20-29

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

15 September 1997

Date of mailing of the international search report

23.09.97

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Authorized officer

Cupido, M

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/01065

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9605292 A	22-02-96	AU 3159995 A CA 2197446 A EP 0778888 A	07-03-96 22-02-96 18-06-97
WO 9510624 A	20-04-95	DE 4335025 A AU 7812094 A EP 0724643 A JP 9503665 T	20-04-95 04-05-95 07-08-96 15-04-97